BS. VET. MED. J. JANUARY 2007 VOL. 17, NO.1, p.50-55

Beni-Suef
Veterinary Medical
Journal

The role of corynebacterial phospholipase D vaccine in activation of macrophages

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The role of phospholipase D toxoid (PLD) vaccine in enhancing killing activity of macrophages was demonstrated in this study. Four groups of Balb/c mice were vaccinated with different forms of current vaccines against *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*). The first group was vaccinated with purified recombinant mutated PLD protein adjuvated vaccine; the second with formalin inactivated whole cells of *C. pseudotuberculosis* adjuvated vaccine, the third group with combined bacterin-toxoid adjuvated vaccine and the fourth was given viable *C. pseudotuberculosis cells*. Mononuclear peritoneal cells from each vaccinated groups were collected and inoculated intraperitoneally into naïve recipient Balb/c mice that were subsequently challenged by equal number of live *C. pseudotuberculosis* cells. Killing activity of peritoneal macrophages collected from each recipient group of mice was assayed by cultivation of lysed macrophages on plates of count brain heart agar. It was reported that the highest killing activity of macrophages were those collected from mice vaccinated with recombinant PLD adjuvated vaccine that reaches 95% of phagocytosed *C. pseudotuberculosis* living bacteria; where those given viable *C. pseudotuberculosis* bacteria (80%); then combined vaccine (69.5%) and the least killing activity was performed by macrophages obtained from bacterin vaccinated animals.

Corynebacterium pseudotuberculosis is a facultative intracellular pathogen that causes caseous lymphadenitis (CLA) in both sheep and goats (Prescott *et al*., 2002) and oedematous skin disease (OSD) in buffaloes (Selim, 2001). CLA vaccines are currently produced from formalin inactivated phospholipase D (PLD)-rich *C. pseudotuberculosis* culture supernatants in which PLD is considered the major protective antigen (Eggleton *et al*., 1991, 2005; Hodgson *et al*., 1999) or from purified recombinant PLD protein inactivated with detergents (Fontaine *et al*., 2006).

Nevertheless the immune mechanisms that involved in protection against *C. pseudotuberculosis* provided by these toxoid vaccines have not yet been clarified. However, the role of humoral immune response resulting in production of anti-PLD antibodies is well established. Neutralization of PLD; the major virulence factor of *C. pseudotuberculosis* enhances bacterial dissemination out of the site of infection in host tissues by increasing vascular permeability, decreasing chemotaxis of neutrophils and inhibiting the opsonic effect of complement through activation of complement cascade by alternative pathway far out from the invasive pathogen (Batey, 1986; Yozwiak and Songer, 1993).

Adequate protection against CLA is afforded by both humoral and cellular immune responses against such intracellular microorganisms (Jolly, 1965; Cameron and Engelbercht, 1971; Hard, 1975; Irwin and Knight, 1975; Cameron, 1982; Lan *et al.,* 1999; Fontaine *et al*., 2006).

Early studies reported the importance of cellular immune response represented by development of activated macrophages that eliminate invasive *C. pseudotuberculosis* from host tissues (Hard, 1969 and Tashjian, 1983). Activated macrophages could be detected in animals infected naturally with CLA or after inoculation of living corynebacteria in experimental animals (Lan *et al*., 1999; Hard, 1969). On the other hand, inactivated *C. pseudotuberculosis* antigen could not stimulate activation of macrophages in immunized animals. Lan *et al*. (1999) reported that activator antigens to

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macrophages are involved in culture supernatants and not cell associated antigens. They reported that these secretory antigens in culture supernatants could stimulate Th-1 subset of $CD4^+$ T cells to produce IFN-ß and TNF-α cytokines; the major activators of macrophages. Factors involved in culture supernatants that could evoke cellular immune response are not previously detected, and the first report about the antigen involved in culture supernatants that can stimulate cell mediated response by *C. pseudotuberculosis* was performed (Walker *et al*., 1994). They identified 40 kDa protein in culture supernatants and investigated its humoral and cellular immune response activities. PLD although considered as the major virulence of *C. pseudotuberculosis* and its role in establishment of humoral immune response is well identified, yet its role in activation of cellular immunity is not elucidated.

Therefore, the present study was undertaken to examine the role of PLD toxoid vaccine in stimulation of cellular immunity and its correlation in production of activated macrophages.

Materials and Methods

Bacterial strain. A locally isolated and completely identified strain of *C. pseudotuberculosis* from clinically infected sheep was kindly provided culture collection at the Biotechnology Center for Services and Research, (BCSR), Faculty of Veterinary Medicine, Cairo University. The isolate is completely identified strain according to standard diagnostic techniques.

Animals. Thirty two 6-weeks-old female Balb/c mice used in the study. Mice were divided into 8 groups; 4 groups, (5 animals/group) that intended for immunization procedures and the other 4 groups (3 animal/group) for macrophage transfer.

Vaccines.

1. Recombinant PLD inactivated vaccine. The PLD toxoid vaccine was prepared by recombinant DNA technology and used for preparation of genetically engineered vaccine against caseous lymphadenitis (CLA) in sheep (Selim, 2000).

2. Bacterin. It is prepared according to Borgden *et al.* (1984). *Corynebacterium pseudotuberculosis* local strain was grown on 0.1% Tween 80 brain heart broth and incubated for 48 h. at 37˚C in shaking incubator. Bacterial cells were collected by centrifugation of culture at 4000 rpm for 15 min. in cooling centrifuge. The pellet was washed twice with distilled water, and then washed once with 50% acetone, once in 100% acetone, twice with ethyl ether and then air dried. The dried pellet was weighed and suspended in 1% formalin solution so that each ml of suspension contained 10 mg of bacterial cells. The formalin suspension was stored in refrigerator for 48 h. Inactivation of bacterial cell was checked by culturing on brain heart agar plates for sterility.

3. *Combined recombinant PLD inactivated protein plus bacterin.* It is composed of toxoid (recombinant genetically inactivated PLD) combined with bacterin (formalin inactivated *Corynebacteria)*. Provided by Prof. S. A. Selim, (BCSR). The components were suspended in oil adjuvant with a concentration of 50 µg recombinant PLD and 10 mg/ml vaccine.

4. *Viable bacteria.* By inoculation of group of mice with viable *C. pseudotuberculosis,* a strain isolated from local sheep breeds diseased with CLA.

Immunization of animals. The first group of Balb/c mice was immunized by 0.2 ml of toxoid subcutaneously and then immunization was repeated once after 4 weeks. The second group was immunized with bacterin in a dose of 0.2 ml. and then given booster after 4 weeks. The third group was immunized with combined vaccine (0.2 ml subcutaneously) and animals were inoculated with booster dose after 4 weeks. The fourth group of animals inoculated once S/C with 0.2 ml live Corynebacteria (10^6 CFU/ml).

Macrophage transfer. Peritoneal macrophages were collected from all groups of vaccinated mice as well as from the unvaccinated control ones. Cells were collected essentially as described by (Cameron and Engelbercht, 1971). The mice were killed with chloroform, the abdominal skin was reflected and 3 ml of tissue culture medium RPMI-1640 containing 10 units of heparin/ml were injected intraperitoneally. The macrophage suspensions from each immunized group were pooled in propylene tubes that were kept in ice to inhibit the adsorption of cells on the walls of the propylene tubes. Macrophages were sedimented by centrifugation at 3000 rpm for 5-10 min., the supernatant was discarded and the collected cell pellets were washed with sterile saline, and then resuspended in non-heparinized RPMI medium using a volume of 0.5 ml for resuspending each pool. The number of collected macrophages was detected by counting the total leukocytic count

Table (1): Killing assay of macrophages collected from Balb/c mice vaccinated with different types of vaccines.

Inoculum type	Mean No. of lysed macrophages from		Mean No. of CFU/100 µl		Percent of killing
	Recipient mice	Control mice	Recipient mice	Control mice	
Toxoid	116.000	116.000	3	58	94.8
Bacterin	972.000	972.000	46	55	16.3
Combined	116.000	116.000	7	23	69.3
Live *C. pseudotuberculosis*	972.000	972.000	11	55	80.0

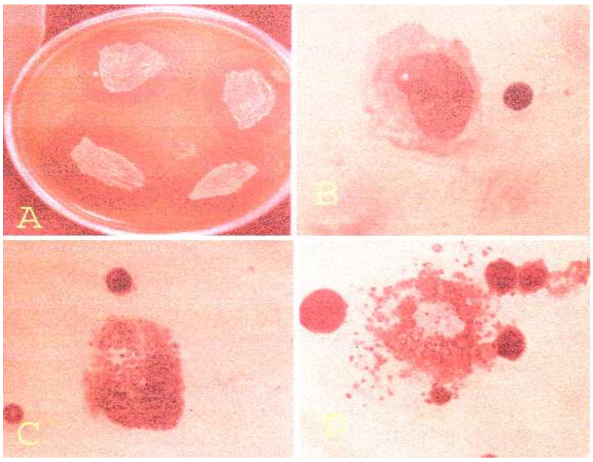


Photo 1: (A) Four strain of *C. pseudotuberculosis* producing PLD showing synergistic haemolysis with *Rhodocoocus equi* filtrate. (B) Naïve macrophage showing minimal phagocytic activity. (C & D) Macrophages collected from mice vaccinated with PLD showing maximal chemotaxis and phagocytosis.

using haemocytometer, then counting the percent of monocytes with Giemsa stain smears prepared from the collected peritoneal fluid. The 0.5 ml of pooled macrophages was adjusted to contain macrophages in a concentration of 6 x 10^6 cell/ml.

Each pool of macrophages was inoculated intraperitoneally into recipient mouse with a dose of 0.5 ml containing 3 x 10^6 cells. All recipient mice were challenged 18 hours later by I/P infection of 0.5 ml suspension of living *C. pseudotuberculosis* containing 2 x 10^6 ml CFU. Control mice (3 mice) were also challenged by the same dose of *C. pseudotuberculosis.*

Counting of live bacteria in the peritoneal macrophages. One hour after I/P inoculation of live *C. pseudotuberculosis,* peritoneal macrophages were collected as previously described but 2 ml saline were used for I/P inoculation and after abdominal gentle massage the peritoneal wash was aspirated and dispensed in ice cooled polypropylene tubes. Ten fold serial dilutions were made using sterile distilled water in the first tubes for lysis of macrophages; the subsequent dilutions were made in sterile saline. Tubes with distilled water were incubated at 37˚C for 15 min. with shaking each 5 min. before making the serial dilutions. Duplicate plate count for each dilution were performed by spreading 0.1 ml of the 10^{-2} and 10^{-3} dilution on brain heart infusion agar plates. The colonies were counted after incubation at 37˚C for 48 h.

Results

The killing assay of macrophages collected from mice vaccinated with toxoid, bacterin, combined (toxoid-bacterin) and living *C. pseudotuberculosis* are illustrated in (Table 1). The highest killing activity was performed by peritoneal macrophages collected from Balb/c mice with adjuvated recombinant PLD vaccine if compared with the same number of macrophages collected from naïve non vaccinated control Balb/c mice (Photo 1). The collected macrophages from recipient mice (116,000 cell) challenged by inoculation of live *C. pseudotuberculosis* and after cultivation of their lysate on brain heart agar media revealed 3 CFU/100 µl of lysate from recipient mice inoculated I/P with macrophages collected from non vaccinated mice as control. The percent of killing of *C. pseudotuberculosis* was 94.8% (Photo 1 C, D). The least killing activity was performed by macrophages collected from mice vaccinated with formalin inactivated whole cell *C. pseudotuberculosis.* The lysate of macrophages revealed 46 CFU/100 µl in comparison with 55 CFU/100 µl of lysed macrophages collected from control mice with a percent of killing activity reached 16.3%. By

addition of PLD toxoid to the bacterin vaccine and using them for vaccination of third group of Balb/c mice, the percent of killing of *C. pseudotuberculosis* raised to reach 69.5 %. Upon vaccination of a fourth group of Balb/c mice with a suitable dose of living *C. pseudotuberculosis* the number of colonies grew from macrophages lysates collected from recipient mice reached 11 CFU/100 µl lysate in comparison to 55 CFU/100 µl of macrophage collected from control mice. The killing percent was 80 %.

Discussion

To develop efficient vaccine against any pathogen the protective antigens and virulence factors must be recognized to know the immune mechanism responsible for protection against infection. The immune response against *C. pseudotuberculosis* is complex and includes both humoral and cellular mechanisms (Lolly, 1965; Cameron and Engelbercht, 1971; Hard, 1969, 1970; Cameron, 1972). PLD is now recognized as the major virulence factor involved in *C. pseudotuberculosis* infections and vaccines prepared by formalin inactivation of culture supernatants rich-in PLD or from purified recombinant PLD exotoxin inactivated with detergents offered significant protection against CLA (Eggleton *et al*., 1991,2005; Burrel, 1983; Fontaine *et al*., 2006). The role of PLD toxoid vaccines in evoking humoral immune response and production of anti-PLD antibodies is adequately documented (Hodgson *et al*., 1994). Nevertheless, the role of toxoid PLD vaccine in stimulation of cellular immunity and activation of macrophages is not previously studied. To elucidate this relation between PLD and macrophage activation, a group of Balb/c mice was vaccinated with purified recombinant PLD antigen inactivated with site directed mutagenesis to avoid any possible drastic effect of formalin on the antigenicity of the PLD antigen. Peritoneal macrophages collected from immunized mice were assayed for their activation by measuring their killing activity to living *C. pseudotuberculosis* bacteria. Results in (Table 1) demonstrated that the average killing activity of macrophages from all vaccinated mice was 95% of phagocytosed cells, which establish a finding that PLD toxoid vaccine could activate phagocytic and killing activity of macrophages. To confirm this finding another group of mice was vaccinated with formalin killed whole cells of *C. pseudotuberculosis* (bacterin). Collected peritoneal macrophages showed no increase in killing activity (18%) that equal the same percentage of killing activity provided by peritoneal macrophages collected from control non vaccinated group of mice. This is in agreement with previous studies reporting that inactivated whole cell vaccines could not stimulate killing activity of macrophages (Cameron and Engelbe-rcht, 1971). Some investigators recommended the formulation of CLA vaccines from toxoid and bacterin as a combined vaccine in believe to improve vaccinal efficacy of each component of administrated in single form (Pointkowski and Shivers, 1998; Fontaine *et al*., 2006).

To evaluate efficacy of combined vaccine in activation of macrophages a third group of mice were vaccinated with combined vaccine composed of the PLD inactivated antigen and bacterin. Macrophages collected demonstrated a killing activity reached 63% which indicated that addition of inactivated bacteria to toxoid vaccine decreased killing activity of macrophages from 95% to 63%. These results coincide with investigation of Eggleton *et al.* (1991) who reported that addition of bacterins did not improve protective efficacy of toxoid vaccines. At the same time did not justify the findings of Pointkowski and Shivers, 1998 and Fontaine *et al*. (2006) in improving immunological efficacy of toxoid vaccine by addition of bacterins.

To prove that activated macrophages are produced as a result of secretory antigens that include PLD, a group of mice was immunized with live *C. pseudotuberculosis*. Macrophages collected from these animals showed a high percent of killing activity that reached 80%, that confirmed the investigation of Lan *et al*. (1999) who attributed activation of cellular immunity to secretory antigens, but not cell associated antigens. These results also agree with data of earlier investigators that reported the activation of macrophages collected from infected animals (Jolly, 1965; Hard, 1969).

The mechanism by which PLD toxoid vaccine could stimulate cellular immunity resulted in production of activated macrophages still needs elucidation, but depending upon the data of the present study and immunologically, it can be proposed that macrophages in case of animals vaccinated with PLD toxoid vaccines were activated due to stimulation of Th-1 subset of

CD4+ T cells which produced cytokines including IFN-β and TNF-α, the patent activators of macrophages (Lan *et al*., 1999) The induction of an adequate Th-1 type T-cell response resulted in production of IFN-β may be an essential component in the induction of cellular resistance to *C. pseudotuberculosis* infection (Tashjian and Campbell, 1983; Simmons *et al*., 1998). The mechanism by which PLD exotoxin activate Th1 lymphocytes is not elucidated and it can be hypothesized that PLD as exotoxin can interact with immune system cells as superantigen. Superantigens are proteins come from microbial sources such as bacteria and viruses. Several bacterial toxins are currently viewed as superantigens.

The staphylococcus exotoxins are the most known, but the pyogenic exotoxin of Streptococci and a protein with mitogenic properties of *Mycoplasma arthridis* are also considered as members of that group (Von Regenmortel *et al*., 1997). Superantigens bind both a specific T-cell receptor VB domain and MHC-II molecules on the antigen presenting cells (APC). They lightly cross link the T-cell and APC resulting in a very powerful T-cell responses which reach 20% of the animal T-cell. This stimulation is associated with the secretion of unusually large amounts of cytokines as IFN-β (Tizard, 2000).

Results of the present investigation provide a conclusion that PLD toxoid vaccines can stimulate both humoral and cellular immune responses which are essential for adequate protection against *C. pseudotuberculosis* infection and it is therefore not surprising that CLA vaccines formulations that focused upon using PLD as the primary protective antigen have been successful (Burrel, 1983; Eggleton *et al*., 1991, 2005; Hodgson *et al*., 1999). On the other hand, the failure of vaccines prepared from inactivated whole *C. pseudotuberculosis* bacterial cells can be explained by lack of development of anti-PLD antibodies that can abolish the toxic effect of PLD secreted by the invasive microorganism and the inability of bacterin in stimulation of cellular immune response and production of activated macrophages the patent cellular factor in elimination of Corynebacteria.

Acknowledgments

The authors acknowledge Prof. S. A.Selim for his valuable scientific advises and his critical revision of the manuscript. The study was financed by Egyptian Academy for Scientific Research & Technology, Grant No. 19.

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دور الفوسفوليبيز- د المحضر من عصيات السل الكاذب فى تحفيز الخلايا البلعمية

فى هذه الدراسة تم إظهار دور اللقاح المحضر من توكسويد الفوسفوليبيز- د (PLD) فى تحفيز النشاط القاتل للخلايا البلعامية. تم حقن أربعة مجموعات من فئران (Balb/c) بلقاحات مختلفة ضد عصيات السل الكاذب. المجموعة الأولى حقنت بلقاح بروتينى منقى (التوكسويد PLD) والمجموعة الثانية حقنت بلقاح عصيات السل الكاذب كاملة ومقتولة بالفورمالين (بكتيرين) والمجموعة الثالثة حقنت بلقاح ثنائى (التوكسويد + البكتيرين) أما المجموعة الرابعة فتم حقنها بعصيات السل الكاذب ، تم تجمع الخلايا أحادية النواة من المحيط البريتونى فى فئران المجموعات السابقة ، و حقنت الخلايا فى مجموعات الفئران المستقبلة ، ثم تلى ذلك حقن مجموعات الفئران المستقبلة بأعداد مساوية من عصيات السل الكاذب الحية كاختبار للتحدى و تم تحليل النشاط القاتل للخلايا البلعمية البريتونية بعد تجميعها من كل من المجموعات المستقبلة عن طريق زرع الخلايا المحطمة منها على أطباق آجار (BHA) و لوحظ أن أعلى مجموعة خلايا لها قدرة على قتل الميكروب كانت المجموعة المحقونة بالتوكسويد (PLD) حيث وصلت إلى ٩٥% تلتها تلك المحقونة بالميكروب الحى كانت ٨٠% بينما كانت النسبة ٦٩,٥% فى المجموعة المحقونة باللقاح الثنائى ، وكانت أقل قدرة للخلايا على قتل الميكروب فى المجموعة المحقونة باللقاح الميت.